# Heat Treatment Adaptations in *Clostridium perfringens* Vegetative Cells†

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## ABSTRACT

Vegetative cells of *Clostridium perfringens* enterotoxigenic strains NCTC 8679, NCTC 8238, and H6 were grown at 37°C followed by a 60-min exposure to 28°C or 46°C. $D_{10}$-values, as a measure of thermal resistance at 60°C, were significantly lower for 28°C exposures as compared with cultures given 37 and 46°C exposures. Following refrigeration at 4°C for 24 h, $D_{10}$-values for the 37 and 46°C samples could not be differentiated from 28°C samples. Western immunoblot analyses of lysates from heat-adapted cells also detected the increased expression of proteins reacting with antiserum directed against the molecular chaperonins from *Escherichia coli*; GroEL, DnaJ, and the small acid soluble protein from *Bacillus subtilis*, SspC. Differential scanning calorimetry (DSC) identified thermal transitions corresponding to ribosomal protein denaturations at 72.1 ± 0.5°C. Any cellular heat adaptations in the DSC profiles were lost following refrigeration for several days to simulate minimally processed food storage conditions. Further analyses of high-speed pellets from crude cell extract fractions using two-dimensional gel electrophoresis detected the differential gene expression of at least four major proteins in heat-adapted vegetative cells of *C. perfringens*. N-terminal amino acid analyses identified two of the proteins as glyceraldehyde 3-phosphate dehydrogenase and rubrerythrin. Both appear to have roles in this anaerobe under stressful conditions.

The safety of mass-produced, low-preservative, minimally processed foods that have overtaken market place deli sections in response to recent consumer demands necessitates studies to decrease potential opportunistic foodborne pathogen outbreaks. The ubiquitous spore-former, *Clostridium perfringens*, is a primary agent of foodborne illness resulting from inadequate heating or refrigeration of prepared foods prior to consumption *(17)*. Symptoms typically occur 8–15 h postingestion of contaminated foods, characterized by abdominal cramps and diarrhea, and are attributed to enterotoxin production upon sporulation of large numbers of vegetative cells in the intestine *(16, 35)*. It is believed that the acidic conditions encountered upon passage through the gastrointestinal tract trigger the sporulation of vegetative cells *(45)*. Increased knowledge of the extent of heat damage to vital pathogen molecules will enhance assessment of food-handling conditions necessary to assure safety.

Stress adaptations add to the concern that marginal food preparation temperatures may not effectively kill all *C. perfringens* bacteria present in food. It has been well documented that nearly all organisms respond to sublethal stress with the production of proteins to counteract and protect against subsequent stress *(6, 19, 39, 44)*. Many of these heat shock proteins are molecular chaperones such as *GroESL* gene products in *Escherichia coli* that aid in proper folding or removal of damaged proteins *(11)*. Rudimentary analyses reported that the thermotolerance of *C. perfringens* increased by two- to threefold following a heat shock of 55°C for 30 min but was maintained for only 2 h following treatment *(13)*. Corresponding proteins were found to be synthesized and were immunologically related to heat shock-induced molecular chaperones, GroEL and DnaK in *E. coli (14)*. The specific function for these proteins in *C. perfringens* cell viability could not be proven directly as they are believed to be a part of a larger general response system to remove damaged proteins *(3, 43)*. Related stress proteins such as the α/β-type small acid soluble proteins (SASPs) from *Bacillus subtilis* have been shown to protect spore DNA from UV damage and could serve a similar role regarding heat damage *(9)*. SASPs have also been shown to exist in *C. perfringens (5, 12, 15)*.

Heat is a very effective means to inactivate foodborne pathogens. Numerous targets of heat damage have been implicated including proteins, enzymes, cellular membranes, and nucleic acids *(26)*. Although DNA melting has been shown to occur at temperatures above 90°C *(23, 42)*, significant damage has also been recorded at much lower temperatures in our laboratory (unpublished results). Bacterial cell death has been attributed to ribosomes, ribonucleic acids, or proteins *(4, 36, 41)*. A single most critical component has not been identified.

Differential scanning calorimetry (DSC) has been used recently by many investigators to examine thermal transitions as indicators of potential sites of cellular injury *(2, 4, 23, 24, 28, 42)*. The technique measures heat transitions as reproducible endothermic and exothermic profiles or thermograms resulting from groups of molecules that react similarly when heated *(4)*. The peaks produced represent major

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components or combinations of components that produce unresolvable profiles at the same transition temperature *(2)*. This study applied DSC to whole cells of *C. perfringens* having increased survivability to sublethal heat stress following exposure to 46°C for 60 min in order to identify critical components of heat damage, such as ribosomes.

The purpose of this study was to test the hypothesis that there are proteins involved in heat stress adaptation of pathogens in addition to those previously described and to test the likelihood of increased pathogen heat resistance following extended refrigeration of precooked foods. A differential two-dimensional protein gel electrophoretic analysis of cell fractions pelleted following ultracentrifugation improved upon earlier heat stress protein studies in *C. perfringens* and characterized additional heat-expressed vegetative cell proteins. The potential for these to be globally heat-regulated could lead to methods for specific enzyme inhibitions to limit pathogen growth in minimally processed foods.

## MATERIALS AND METHODS

**Microorganisms and chemicals.** Only enterotoxin-producing strains of *C. perfringens* were used in this study. *C. perfringens* strain H6 (Hobbs serotype 6) was obtained from the Centers for Disease Control (Atlanta, Ga.). *C. perfringens* strains NCTC 8238 (Hobbs serotype 2) and NCTC 8679 (Hobbs serotype 6) were obtained from Dr. O. Giger at the University of Wisconsin (Madison, Wis.). The vegetative cell growth medium consisted of fluid thyoglycolate (FTG) medium (Difco Laboratories, Detroit, Mich.). All biochemicals used for molecular analyses were purchased from Sigma-Aldrich Chemical Co., Inc. (St. Louis, Mo.) Rabbit antiserum raised against *B. subtilis* SASP, SspC, was provided as a generous gift from Dr. Peter Setlow at the University of Connecticut (Framingham, Conn.). Rabbit polyclonal antibodies raised against *E. coli* chaperonins GroEL and DnaJ were purchased from StressGen Biotech. Corp. (Victoria, B.C., Canada).

**Vegetative cell preparations for thermal inactivation studies.** Freshly steamed 10-ml FTG medium tubes were inoculated individually to a final concentration of $10^5$ viable spores/ml from spore suspensions originally at $10^8$ CFU/ml, prepared as previously described *(8)*, and heat-shocked at 75°C for 20 min prior to growth. The FTG tubes were incubated at 37°C for 4 h with spectral absorbance readings taken every hour at a wavelength of 600 nm. Mid-log phase cultures were then transferred to 28, 37, or 46°C for 60 min. All vegetative cells were placed on ice prior to sampling. All heat inactivation experiments were conducted in bacteriological medium (FTG). No spores were detected microscopically. The harvested vegetative cells were either used directly (controls) or refrigerated at 4°C for 24 h prior to initiating studies (treated cells). Cells were plated before and after refrigeration to evaluate viability changes due to refrigeration. Experimental data were collected following heat treatment at 60°C using a submerged coil heating apparatus *(7)*. Samples (0.2 to 1.0 ml) were removed at predetermined time intervals and rapidly cooled on ice prior to enumerations of survivors. Mid-log phase cultures of *C. perfringens* vegetative cells transferred to 28 and 46°C were also pelleted at 5,000 × *g* and washed with peptone-water (0.1%) (wt/vol). These wet pellets were then either loaded into aluminum pans for DSC directly or refrigerated at 4°C prior to DSC.

**Enumeration of survivors.** In order to determine the number of surviving CFU/ml, samples were serially diluted in 0.1% (wt/vol) peptone-water and plated on Shahidi Ferguson perfringens agar base medium (Difco) using a spiral plater (Spiral Biotech, Bethesda, Md.; model D). After 30 min, a Shahidi Ferguson perfringens agar overlay was added to the plates and incubated at 37°C for 18 h under anaerobic conditions (85% $N_2$, 10% $CO_2$, and 5% $H_2$), followed by enumeration of surviving cells.

**Calculation of *D*-values.** $D_{10}$-values (defined as the time in minutes at a specified constant temperature necessary to reduce the viable cell population by 1 $\log_{10}$ or 90%) were determined by plotting the $\log_{10}$ number of survivors against time at 60°C using Lotus 1-2-3 software. The line of best fit for survivor plots was determined by regression analysis *(33)*; a regression equation for the type $y = a + bx$ was derived, where $b$ is the slope of the best straight line and, when inverted and the sign changed from − to +, gives the $D_{10}$-value in minutes for the specific temperature (60°C in this study). Only survival curves with more than five values in the straight portion, with a correlation coefficient ($r^2$) > 0.90 and descending more than 6 log cycles were used. The heat-resistance data were analyzed by analysis of variance using the Statistical Analysis Systems (SAS) *(38)* to determine whether there were statistically significant differences among the treatments.

**Whole vegetative cell lysates.** Vegetative cells were prepared for denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis immediately following harvest. Cell pellets were resuspended in gel loading buffer *(37)* and lysed at 100°C in a boiling water bath for 5 min. These were then stored at −70°C until needed. Duplicate samples were resuspended in similar volumes of sterile distilled water for quantitation of total protein using a revised Lowry method *(21, 25)*.

**Gel electrophoresis and immunoblot analysis.** Proteins were electrophoresed using standard SDS-PAGE procedures (4% [wt/vol] acrylamide stacking gel, 15% [wt/vol] acrylamide resolving gel; 30:0.8 acrylamide: bis acrylamide) and a modified Tris-glycine buffer system *(22)*. For immunoblots, denatured proteins were transferred to Immobilon-P polyvinylidene difluoride membranes (Fisher Scientific, Pittsburgh, Pa.) using a Panther semidry electroblotting apparatus model HEP-1 (Owl Separation Systems, Portsmouth, N.H.). Blots were prewashed with phosphate-buffered saline (PBS)-0.05% (vol/vol) Tween-20 (PBS-Tween) as previously described *(37)*. Dehydrated nonfat milk (5%) (wt/vol) in PBS-Tween was used as a blocking agent overnight. Antibodies raised against specific proteins were diluted in PBS-Tween as follows: α-SspC (1:3,000), α-GroEL (1:5,000), and α-DnaJ (1:3,000). The blots were incubated with agitation for 1 h at room temperature *(31)*. A colorimetric detection assay was used with secondary antibody consisting of goat anti-rabbit IgG conjugated to alkaline phosphatase (Gibco BRL Life Technologies, Grand Island, N.Y.) that was diluted 1:3,000 in PBS-Tween and incubated for 2 h prior to chromogenic substrate development *(37)*.

**Cellular fractions for DSC and two-dimensional gel electrophoresis.** Cellular components were prepared according to the following modifications on previous methods reported *(23)*. Cell pellets from 3 liters of mid-log phase *C. perfringens* vegetative cells were spun down at 5,000 × *g* for 10 min. The pellet was washed and resuspended in 15 ml of ribosome suspension buffer consisting of 25 mM Tris (pH 7.5), 1 mM EDTA (pH 7.5), 5 mM β-mercaptoethanol, 6 mM $MgCl_2$, and 30 mM $NH_4Cl$ *(34)*.

The cell pellets were then stored at −70°C until use. The protease inhibitor, phenylmethylsulfanylfluoride, was added to 1 mM as was dithiothreitol to provide a stable reducing environment prior to cell lysis. Cells were broken by two successive passes

through a French pressure cell at 12,000 psi. DNAase was then added to a concentration of 0.2 mg/ml. Centrifugation at 32,500 × *g* for 30 min produced a pellet of broken cells and membranes. The remaining supernatant was then centrifuged at 150,000 × *g* for 3.5 h in a Beckman XL-70 ultracentrifuge with a 70 Ti rotor (Beckman Instruments, Palo Alto, Calif.). From previous studies, the pellet at this stage was believed to consist primarily of crude ribosomes *(34)*. The supernatant formed the soluble crude cellular extract. All of the high-speed crude cell extract fractions were frozen at −70°C prior to loading into aluminum pans for DSC.

**DSC.** Weighed samples (12 to 20 ± 0.1 mg wet weight) were hermetically sealed in 20-ml aluminum DSC pans. Reference pans contained sterile peptone-water for whole cells or ribosome suspension buffer for cell fractional components. Samples were cooled to 10°C and allowed to equilibrate for 2 min prior to heating to 100°C at a rate of 10°C/min. The calorimeter used was a Perkin-Elmer Pyris I (Perkin-Elmer Corp., Norwalk, Conn.) calibrated with indium and cyclohexane. Data were collected and analyzed using Pyris I software. Only those peaks that were present in multiple scans were selected for analysis. Average peak temperatures were calculated from multiple thermograms for each sample.

**Two-dimensional gel electrophoresis.** Two-dimensional gel electrophoresis *(32)* was performed using a Protean II xi 2-D cell and reagents according to protocols of Bio-Rad Laboratories (Hercules, Calif.). The first dimension involved isoelectric focusing on 4.5% acrylamide tube gels (13 cm long, 1.5 mm wide) with a linear pH gradient from 3 to 10. The cathode electrolyte buffer consisted of 0.02 N NaOH, whereas the anode electrolyte buffer comprised 0.01 N $H_3PO_4$. Electrophoresis running conditions were held constant at 200 V for 1 h, then 500 V for 1 h, followed by 560 V for 16 h. The second dimension consisted of SDS-PAGE on 12% acrylamide slab gels under constant current at 25 mA for 10 h. Gels were loaded with 200 μg of protein and visualized using the Bio-Rad Silver Stain Plus kit. For N-terminal amino acid analyses, protein spots were localized on polyvinylidene difluoride membranes following semidry transfer in 10 mM 3-(cyclohexylamino)-1-propane-sulfonic acid, pH 11.0 buffer with 10% methanol using the Panther electroblotting apparatus (Owl Separation Systems), Coomassie staining of the polyvinylidene difluoride membrane, and excision according to standard protocols obtained from the Molecular Structure Facility (University of California at Davis, Calif.). N-terminal protein sequence analyses of protein spots were performed by using Edman degradation coupled with on-line high-performance liquid chromatography and data acquisition by the Molecular Structure Facility at U.C. Davis.

## RESULTS

**Cell adaptations to temperature.** Vegetative cells of *C. perfringens* strains NCTC 8679, NCTC 8238, and H6 were found to be capable of growth at 37°C to mid-log phase and a culture optical density at 600 nm of 0.688 in 3 h. Following separate incubations at 28, 37, and 46°C for 60 min, vegetative cell $D_{10}$-values for 60°C are listed in Table 1. $D_{10}$-value effects, as a measure of thermal resistance for each of the enterotoxigenic strains, increased following controlled temperature elevations above 28°C (Table 1). Increasing the temperature from 37 to 46°C did not significantly increase the effect (Table 1). To determine whether the temperature adaptation could be maintained over the course of a typical refrigerated food-storage period, heat-adapted cells were treated at 4°C for 24 h prior to quantifying the heat resistance at 60°C. Viability changes as a result of the 24-h refrigeration were determined to be insignificant for each strain tested, decreasing from 6.89 ± 1.10 CFU/ml to 6.70 ± 0.57 CFU/ml for NCTC 8679, 6.75 ± 0.14 to 6.64 ± 0.15 CFU/ml for NCTC 8238, and from 6.84 ± 0.08 to 6.32 ± 0.08 CFU/ml for H6. However, the stored refrigeration, in every sample tested, did result in a significant loss of initial temperature adaptation (Table 1).

TABLE 1. *Thermal inactivations of* C. perfringens *vegetative cells at 60°C using a submerged coil heating apparatus*

| Strains | 60-min incubation temperature (°C) | Corresponding $D_{10}$-value[a] | 4°C storage $D_{10}$-value[b] |
|---|---|---|---|
| NCTC 8679 | 28 | 1.93 ± 0.22 A | 1.95 ± 0.97 AC |
| NCTC 8679 | 37 | 3.75 ± 0.40 B | 2.92 ± 0.07 C |
| NCTC 8679 | 46 | 3.86 ± 0.18 B | 1.49 ± 0.42 C |
| NCTC 8238 | 28 | 2.29 ± 0.23 A | 2.24 ± 1.30 AC |
| NCTC 8238 | 37 | 3.04 ± 0.13 B | 2.34 ± 0.18 C |
| NCTC 8238 | 46 | 4.05 ± 1.38 B | 3.06 ± 0.16 C |
| H6 | 28 | 2.61 ± 0.47 A | 2.35 ± 0.39 AC |
| H6 | 37 | 4.22 ± 0.95 B | 1.88 ± 0.17 C |
| H6 | 46 | 3.32 ± 0.32 B | 2.28 ± 1.16 C |

[a] $D_{10}$-values are the average of two separate experimental trials immediately following temperature incubation. Means with the same letter are not significantly different ($P < 0.05$).

[b] These vegetative cells were first stored for 24 h at 4°C prior to $D_{10}$-value evaluation.

The protective, adapted tolerance to elevated temperature was most evident after treatment at 37 and 46°C for each strain. $D_{10}$-values for *C. perfringens* strain NCTC 8679 increased from 1.93 min for the 28°C exposure to 3.75 min for the 37°C exposure, and 3.86 min for the 46°C exposure (Table 1). This represented a twofold increase in heat resistance at 60°C for vegetative cells after 37 and 46°C exposures as compared to 28°C. However, $D_{10}$-values following refrigeration at 4°C for 24 h for the NCTC 8679 cultures significantly decreased to approach levels approximating that obtained for the lowest temperature exposure (Table 1). *C perfringens* strain NCTC 8238 exhibited small $D_{10}$-value incremental increases at 2.29 min for the 28°C sample, 3.04 min for the 37°C sample, and 4.05 min for the 46°C sample exposure (Table 1). If the samples were refrigerated at 4°C for 24 h prior to $D_{10}$-value determinations, heat resistance properties for the 37 and 46°C exposed vegetative cells again significantly decreased (Table 1). *C. perfringens* strain H6 exhibited $D_{10}$-value and heat resistance increases in response to temperature adaptation covering a range of 2.61 min for 28°C, 4.22 min for 37°C, and 3.32 min for 46°C, respectively. Upon prior refrigerated storage $D_{10}$-values and measured thermal resistance at 60°C for H6 dropped from 2.61 to 2.35 min for the 28°C treatment, 4.22 to 1.88 min for the 37°C sample, and from 3.32 to 2.28 min for the 46°C sample treatment (Table 1).

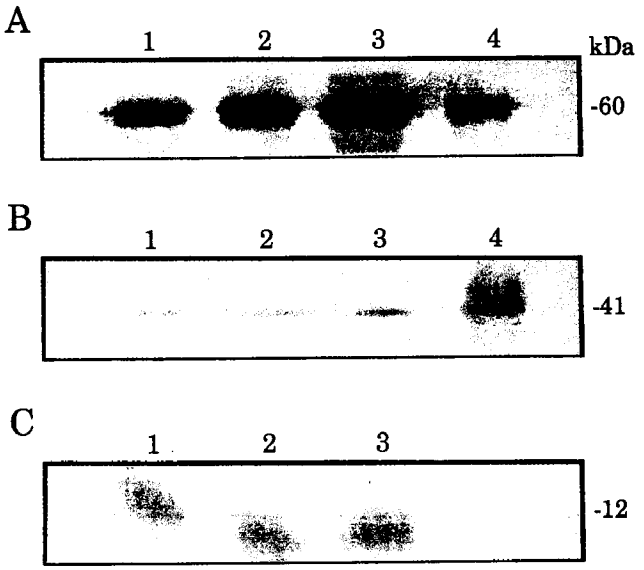


FIGURE 1. *Western immunoblots of whole cell lysates obtained from* C. perfringens *strain H6 mid-log phase vegetative cells following temperature adjustments for 60 min. All sample lanes were loaded with 10 μg of total protein. Immunoblots (A) and (B) were reacted against antibodies for* E. coli *GroEL and DnaJ, respectively. Blot (C) was reacted against antiserum raised against* B. subtilis *SspC protein. Lanes: 1, 28°C (nonheat-stressed control); 2, 37°C; and 3, 46°C; 1A, lane 4,* E. coli *GroEL standard (60 kDa); and 1B, lane 4,* E. coli *DnaJ standard (41 kDa).*

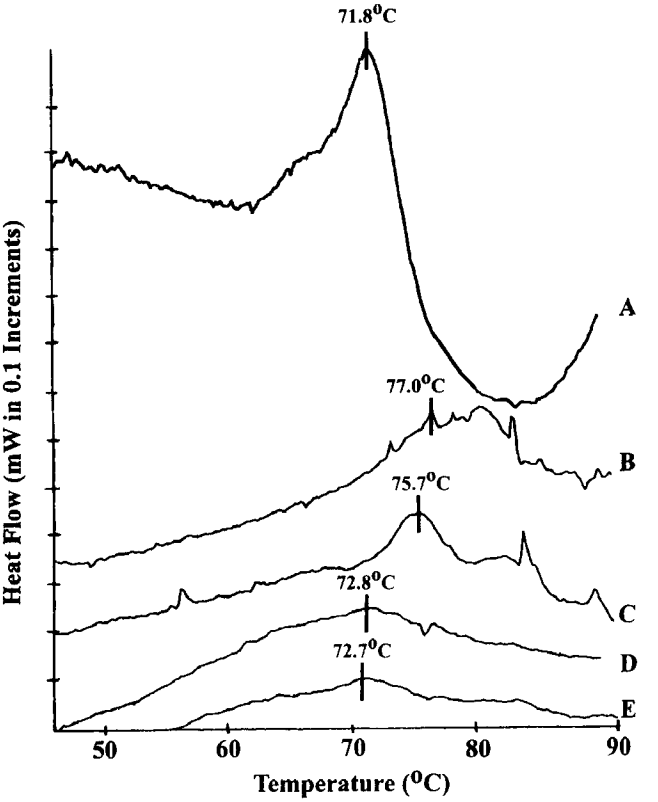


FIGURE 2. *DSC of* C. perfringens *strain H6 vegetative cells grown in FTG medium at 37°C to mid-log phase. Representative thermograms from three separate trials for each condition: A, ribosomal protein fraction-control (14.84 mg); B, cells treated at 46°C for 60 min (20.00 mg); C, cells treated at 28°C for 60 min (18.10 mg); D, B followed by extended storage at 4°C (16.85 mg); and E, C followed by extended storage at 4°C (14.00 mg).*

**Temperature-adapted vegetative cell protein expression.** Representative Western immunoblot analyses of the *C. perfringens* vegetative cell lysates from enterotoxigenic strains are depicted in Figure 1. A 60-kDa band is seen with antigenic similarity to *E. coli* GroEL and is shown to increase in density as controlled temperature exposure is also increased (Fig. 1A). Likewise, a 41-kDa protein band with antigenic similarity to *E. coli* DnaJ is evident with increased expression or decreased turnover in response to increasing temperature exposure (Fig. 1B). A very prominent diffuse protein band is present at approximately 12 kDa that immunoreacts with *B. subtilis* SspC antibodies *(Fig. 1C).*

After the 4°C temperature storage for 24 h, proteins drop below detectable levels using Western immunoblot analyses for each of the strains (data not shown). This corresponds to diminished protection against thermal inactivation as has been shown by decreased $D_{10}$-values at 60°C following prolonged storage (Table 1). However, total cell viability did not significantly decrease following the 24 h storage at 4°C in FTG culture medium and was similar for each of the strains examined as previously described.

**DSC cell fractionation studies.** Using DSC, heat absorbed by similar molecules is represented by positive endothermic peaks while heat expended results in negative exothermic peaks. Characteristically, proteins are irreversibly denatured as a result of the heating process and thermogram profiles disappear upon reheating. None of the individual cellular fractions exhibited any thermal transitions above background with the exception of the high speed-pelleted, crude cell extract fraction (Fig. 2). This concentrated ribosome fraction produced a broad endotherm (average of three trials = 72.11 ± 0.51°C) with a modest shoulder (Fig. 2A). Upon reheating, the endothermic peak disappeared, proving that the proteins were irreversibly denatured during the initial heating exposure (unpublished data). It is possible that only the ribosomal proteins were in high enough concentration to be accurately detected during this study.

**DSC whole cell studies.** Whole cells from *C. perfringens* strains exposed to elevated temperatures of 46°C for 60 min were compared to control cells kept at 28°C for 60 min in relation to differences in DSC thermogram transitions (Fig. 2B and 2C). It has been shown that vegetative cell $D_{10}$-values increased for 46°C-exposed cells as compared with 28°C-exposed cells along with increases in protein expression (Table 1 and Fig. 1). Indeed changes were apparent in the DSC thermograms obtained for *C. perfringens* cells exposed to 46°C as compared with 28°C (Fig. 2B and 2C, respectively). A very broad endothermic peak at 77.0°C for the 46°C sample was a shift to higher temperatures as compared with the 28°C-treated sample (75.7°C) that exhibited a thermogram profile nearer to that from the isolated whole ribosomal fraction (71.8°C) (Fig. 2A and 2C).

As an indication of the stability of the observed thermal effect during prolonged storage at 4°C, as might be

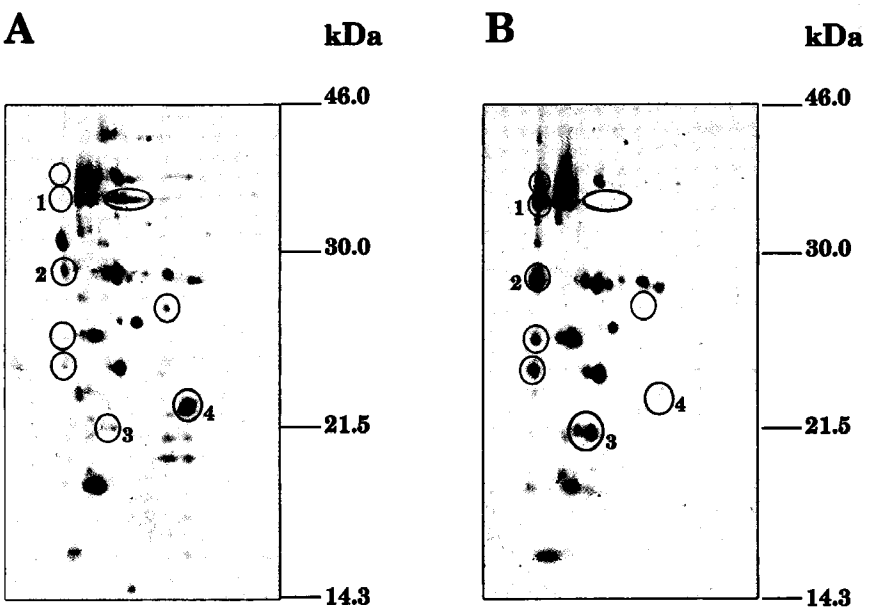


FIGURE 3. *Two-dimensional protein profile of the ribosomal fraction of proteins expressed in* C. perfringens *strain H6 vegetative cells grown at 37°C followed by exposure to A, 28°C for 60 min; and B, 46°C for 60 min. Circled areas with numbers denote proteins differentially heat-regulated that were isolated for subsequent transfer to membranes and N-terminal amino acid sequence analysis. The proteins (200 μg total) were resolved on pH 3 to 10 linear pH gradient 4% acrylamide tube gels in the first dimension. The second dimension consisted of SDS-PAGE using 12% acrylamide slab gels.*

encountered in a supermarket deli case or consumer refrigeration of leftovers, samples were also analyzed after refrigeration for several days following the 46 and 28°C treatments. Both whole cell samples produced ribosome-associated similar reduced broad endotherms at 72.8 and 72.7°C, respectively (Fig. 2D and 2E). As a result, the stability of the proteins in the high speed crude cell extracts in response to temperature changes was investigated further.

**Two-dimensional protein gel analysis of heat adaptations.** High speed-pelleted crude cell extract fractions, as opposed to total cell lysates, were compared electrophoretically from *C. perfringens* cells exposed to 28 and 46°C, respectively (Fig. 3A and 3B). This enabled a clearer visualization of differentially expressed proteins in the crude ribosomal preparations used for the DSC results (Fig. 2). Following exposure of cells to 46°C for 60 min, protein spots numbered 1, 2, and 3 are significantly increased in density, whereas spot number 4 is decreased (Fig. 3B). Several other protein differences were noted, but these were determined to be most apparent and therefore selected for transfer to polyvinylidene difluoride membranes and identification using N-terminal amino acid sequencing.

Low concentration and resolution limits prevented the identification of spot 1. The N-terminal sequence analysis of spot 2 resulted in the sequence SVKVAINGFGRI and was found to have 100% identity for a 12 N-terminal amino acid overlap in glyceraldehyde 3-phosphate dehydrogenase (Swiss-Prot search) *(27).* The 35-kDa molecular weight reported for this protein is higher than the 30 kDa approximated for spot 2 (Fig. 3). The N-terminal sequence analysis of spot number 3 resulted in the sequence KTLKGXXTAENXA and was found to have 72.7% identity with an 11-amino acid overlap at the N-terminal end of rubrerythrin in *C. perfringens (18).* The reported molecular weight of 22 kDa is in good agreement with results in Figure 3. The N-terminal analysis of spot 4 was not attempted as the protein was degraded or synthesis was terminated following heat treatment. Nearly all selected proteins from the ribosomal preparation were also found to migrate in the neutral range of the 3 to 10 pH gradient of the first dimensional gel run. Expression of these proteins may be involved in adaptation to heat treatment.

## DISCUSSION

The Food Safety and Inspection Service of the U.S. Department of Agriculture recently issued stabilization performance standards for preventing the growth of spore-forming bacteria to the limit of no more than a $\log_{10}$ multiplication of *C. perfringens* in foods. *(10).* Complete heat sterilization of foods to completely eliminate spore formers is impractical with respect to the nutrient and organoleptic integrity of the foods. We chose to initiate this study on the vegetative cell heat adaptations of *C. perfringens,* acknowledging the increased heat resistance of the spore, but concurrently recognizing the real cause of this foodborne disease as the propagation of large numbers of vegetative cells in temperature-abused, minimally processed foods. The molecular responses of *C. perfringens* enterotoxigenic strains NCTC 8679, NCTC 8238, and H6 were examined following heat adaptation. It has been well established that bacteria have the ability to adapt to various nonlethal stresses *(1).* Stress adaptations warrant further study when establishing minimal guidelines for food safety and processing.

The vegetative cells of *C. perfringens* acquired an increased resistance to heat corresponding to increased levels of specific proteins at elevated temperatures. With respect to $D_{10}$-value increases, the heat resistance of the strains acquired during the 28 to 37°C exposure did not significantly increase at temperatures above 37°C. However, GroEL and DnaJ proteins increased with elevated temperature exposure for all vegetative cell strains examined. Following a 60-min heat shock, the thermal effects were lost upon refrigerated storage at 4°C for 24 h. This adds support to an earlier study where elevated heat resistance effects were found to be transient *(13).* Although heat shock proteins had been previously shown to be expressed in *C. per-*

*fringens (14),* this study gives additional evidence with greater detail for DnaJ and *B. subtilis* SspC similar proteins, as well as emphasizes ribosomal protein changes. It has been suggested that heat shock proteins synthesized temporally in vegetative cells even increase spore heat resistance by altering spore structure *(30).*

It was unexpected that vegetative cells of *C. perfringens* were found to contain proteins that immunologically reacted with *B. subtilis* SspC antiserum. Previously, SASPs had only been identified in *C. perfringens* spores *(5, 12, 15).* The absence of spores was verified microscopically as well as by direct plating on Shahidi Ferguson perfringens medium with overlay following heat shock at 75°C for 20 min to kill vegetative cells. However, *C. perfringens* enterotoxin has also been reported to exist in vegetative cells prior to sporulation, and this may represent another incidence of leaky gene regulation in *C. perfringens (20).*

Another peculiarity was the report of identified *C. perfringens* SspC1 and SspC2 proteins similar to *B. subtilis* α/β type SspC SASP with molecular weights of 6.3 and 6.6 kDa, respectively *(15).* The primary SASP-like product visualized on Western blots in this study had an approximate molecular weight of 12 kDa following gel electrophoresis under denaturing conditions. It is possible that the size difference may be attributed to slower electrophoetic gel migration of glycoproteins *(12),* or the cross reaction of the antiserum with another SASP-like protein. It would be very intriguing if the 12-kDa protein was a SASP in that SASPs have been shown to protect DNA from UV irradiation damage *(9)* and also protease digestion *(40).* A similar occurrence would be anticipated regarding thermal damage.

It is believed that heat is uniformly distributed in a cell resulting in damage to only the most sensitive molecules within that cell *(29).* An attempt was made in this study to identify a critical component responsible for the heat sensitivity limits of *C. perfringens* using DSC. The technique was first used to combine measurable heat resistance of microorganisms with the thermostability of specific cell components *(28).* During heating of the cells, a sequence of transitional events is believed to cause perturbations to the specific heat capacity of the cell *(4).* As a result, characteristic patterns of positive (endothermic) and negative (exothermic) transitions are recorded *(4).* However, there is an inherent difficulty in making precise correlations regarding recorded thermograms from purified fractions and conditions in whole cells under physiological conditions *(23).* Alternative explanations may be given for the major transitions observed in a thermogram including that the peak may represent a composite of many minor transitions, a single component is present in high concentration, the combination of strongly interacting components, or a set of coupled transitions *(4).*

Ribosomes are known to contain approximately 40% protein as well as comprise a majority of the dry weight of the bacterial cell *(42).* Therefore, it is not surprising that the only identifiable endotherms present in the thermograms produced from whole cells matched the ribosomal fraction of the high speed crude cell extracts. The 28°C sample produced an endothermic peak at 75.7°C, whereas the 46°C sample had a slightly elevated endotherm at 77.0°C. Refrigeration of the cells at 4°C for 24 h restored endotherms to those represented by the concentrated ribosome control fractions (72.1 ± 0.5°C). This series of experiments suggested conformational changes in ribosome proteins in response to temperature differences that might alter the efficacy of protein synthesis. As a result, the high speed-pelleted crude cell extract fractions of the cells were separated for subsequent two-dimensional electrophoretic analyses.

Four specific numbered locations on the two-dimensional gels were determined to contain proteins with expression profiles regulated in response to a cell growth temperature elevation from 28 to 46°C. Increases in protein expression with temperature elevation were evidenced for spots 1 to 3 (molecular weight: 35, 30, and 22 kDa, respectively), whereas spot 4 disappeared. None of these were in the size range that would be expected for the heat shock proteins analyzed by Western blots previously (60, 41, or 12 kDa).

The resolution limitations of the two-dimensional system used prevented reliable isolation of protein 1 for subsequent N-terminal amino acid analyses. Protein 2 was identified with a high degree of certainty as glyceraldehyde 3-phosphate dehydrogenase *(27).* This enzyme plays a central role in the glycolytic pathway of anaerobic metabolism. It is not unwarranted to expect an increased presence of the enzyme in response to temperature stress. The 30-kDa size of glyceraldehyde 3-phosphate dehydrogenase in *C. perfringens* might represent a species variation or possible post-translational modifications. Protein number 3 was identified as rubrerythrin, a protein in *C. perfringens* suspected to play a role as a scavenger of oxygen radicals under stressful conditions *(18).* Protein 4 was not analyzed further as it might have been turned over or degraded at a higher rate following temperature elevations. It is not clear whether the differences in protein levels exhibited are at the transcriptional or translational levels of regulation. However, the differences do coincide with notable differences in heat resistance of the vegetative cells (Table 1). They are obviously not exclusive but most likely associated with other as yet unidentified proteins involved in the global regulation of heat resistance in the cells of *C. perfringens.*

In the search for the crucial protein that is the rate-limiting primary target in heat killing, the current belief is that membrane proteins may be denatured by heat initially because of peripheral locations followed by the denaturation of crucial proteins within ribosomes *(4).* If these proteins are vital to cell survivability, it would seem reasonable that protective repair mechanisms would be needed. Heat adaptation must involve synthesis of some proteins of which ribosomes would certainly be expected to play a significant role.

With respect to food safety regulations and a single critical component of thermal damage within foodborne pathogens, it still is important to remember that heat induces varying degrees of injury within cells resulting in death or recovery depending on the degree of injury and the conditions under which the cells are held *(29).* In the

present study, a number of heat shock proteins were found to be associated, the stability of which might be important for survival of *C. perfringens* during thermal processing. The identification of differentially expressed proteins will enable future enzyme or structural protein characterizations that may be applied to food formulations in order to better control the metabolism and growth of foodborne pathogens.

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